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cGAS is activated by DNA in a length-dependent manner

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 February 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees acknowledge the potential interest of the findings. Nevertheless, they have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here, but I think it will be of particular importance to use additional (or other) types of PCR templates (non-bacterial) - as suggested by all three referees -, maybe also synthetic oligos, and additional delivery methods, ideally electroporation. Let me also point out, as EMBO reports emphasizes novel functional over detailed mechanistic insight, we would not require additional experiments to provide further insight into cGAS length discrimination mechanisms.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in a point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

In their manuscript titled "Length-dependent sensing of low-abundance dsDNA by cGAS", Luecke and colleagues investigate the relation of linear double-stranded DNA-length and its potency as a ligand for the cytosolic cGAS-STING pathway. They demonstrate that -when transfected at low concentrations with Lipofectamine 2000 - increased length of PCR-products leads to increased type I IFN production. Using mainly the human monocytic THP-1 cell line as a model system, they demonstrate that the recognition is dependent on cGAS and partially on IFI16 and claim that the observed length-specific difference is unaltered in TREX1-deficient cells. While length-dependent activation of cytosolic dsDNA-sensing has been known long before the discovery of cGAS (Stetson & Medzhitov, 2006), previous studies have focused on minimal length requirements rather than differences in a higher length spectrum. The authors furthermore claim that the observed length-discrimination is an intrinsic property of cGAS and provide an in vitro assay of cGAMP production. Despite this claim, no mechanistic explanation of length discrimination is investigated.
The finding in itself is of interest to the scientific community, however the manuscript in its current state does not sufficiently address possible experimental problems and alternative explanations. Only when these issues are addressed, the manuscript can be considered for publication.

Major concerns:

1. Choice of DNA ligands (applies to all experiments)

The authors use PCR products of different lengths to investigate the length-dependency of the DNA-response. Using solely this technical approach poses several problems.

1.1. The authors use a silica-column based purification approach to isolate PCR-products. This purification method can introduce a purity bias against smaller products. Usually, the amount of primers and dNTPs carried over is proportional to the amount of PCR reaction used, while the same volume of PCR reaction yields a higher mass of PCR product for higher lengths. This means that the same mass of a smaller PCR product will most likely have a higher contamination with primers and dNTPs, which cannot be distinguished in standard photometric quantification. The authors should carefully assess this by appropriate quantification methods (e.g. PicoGreen) and more importantly using other DNA sources, like synthetic oligonucleotides and plasmid DNA fragments(see also 1.3).

1.2. Only one PCR-template is used. While this is a sound approach for an initial test, the resulting PCR-products may strongly vary in local and overall GC-content as well as PCR-efficiency and thereby purity (see 1.1.). The authors should at least include 2 representative additional PCR-products for each length, preferably with low and high GC content each, to assess the general applicability of the observed effects.

1.3. Only PCR-products are used. The short PCR-products should be complemented with annealed synthetic oligonucleotides of defined length, also to address the concerns named in 1.1. Longer fragments should be added in the form of linearized as well as circular plasmids, which are readily available in the range of 3 to 20 kb in most laboratories. Plasmids digested with 4-mer cutters could also serve as a more reliable source of mixed fragments between 100 and 500 bp.
The authors furthermore argue that the observed effect may be important for the recognition of for instance the HSV-1 genome, which is very large. DNA of comparative size (approx. 150 kb) is also easily obtainable from bacterial artificial chromosomes, and thus should be included in the analyses.

2. Choice of delivery method (applies to all experiments involving transfection)

2.1. Lipofectamine 2000 is used throughout the manuscript. All transfection reagents have a unique size bias depending on their mode of action and chemical properties. The authors should confirm their results with other reagents of different classes or generations (PEI, GeneJuice, Lipofectamine 3000). Reagents designed for small fragments like Lipofectamine RNAimax would be especially recommendable.

2.2. The transfection efficiency determination in Fig. 3A does not distinguish cGAS-available free cytosolic DNA from membrane-bound or intracellular intact Lipofectamine-DNA-particles. In fact, free DNA would have a very short half-life in the cytosol due to TREX1-mediated degradation. Therefore, a higher signal for the shorter PCR product could even suggest a delivery disadvantage for shorter DNA.

2.3. The authors should use electroporation as a relatively unbiased and less "sticky" delivery method.

3. Role of TREX1 and general strength of the observed effects (applies to Fig. 3 B in comparison to other stimulations).

In most datasets, the authors use a small PCR product set of 94, 500 and 4003bp. In their laboratory THP-1 cell lines, a robust difference between the three PCR-products can be observed. Using TREX1-deficient THP-1 cells and respective control cells from Invivogen the authors claim that the observed differences are independent of TREX1. However, the difference between 500 bp and 4003 bp is barely visible in either TREX1-/- or WT cells, and also the response to the 94 bp fragment is only reduced by about 50 % in comparison to the 500 and 4003 bp fragments. The figure in its current form does not convincingly substantiate the claim that TREX1 is dispensable for the observed difference.

4. cGAS in vitro assay (applies to Figure 3 D, E and EV3 C).

4.1. The general concern about the purity and accurate quantification of short PCR-products (1.1) applies here especially. The short PCR product will very likely have a higher contamination with primers and dNTPs than the longer ones. Apart from using a lower PCR-product concentration than intended and measured, primers might interfere with the reaction by non-productive binding of cGAS. Also, dATP from dNTPs can inefficiently be used as cGAS substrate in vitro (Gao et al., 2013), possibly leading to inhibition of cGAMP formation by competition. The authors should definitely repeat these experiments with defined fully synthetic short ligands replacing the 94 bp PCR-product and use linearized plasmid instead of or in addition to the 4003 bp PCR.

4.2. In EV3 C, the authors highlight "degradation products". What does this refer to? What is degraded? The lowest peaks for degradation products coincide with highest apparent cGAMP production. How is this related?

Gao, P., Ascano, M., Wu, Y., Barchet, W., Gaffney, B. L., Zillinger, T., et al. (2013). Cyclic [G(2',5')pA(3",5")p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. Cell, 153(5), 1094-1107.

Stetson, D. B., & Medzhitov, R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity, 24(1), 93-103.

Referee #2:

In this manuscript the authors examine the innate immune response to exogenous (transfected) DNA, and find that DNA sensing is length-dependent (from <100 to ca 4000 bp), and this is due to the cytosolic DNA receptor cGAS. This is a relatively incremental advance to the current knowledge in the field, given that DNA sensing is known to be largely length-, rather than sequence-dependent, and cGAS is widely recognised as the major cytosolic DNA receptor. The additional information provided by this study is that the length-dependence extends to longer DNA fragments (100s and 1000s bp), with shorter fragments requiring a higher concentration to be detected. The authors also show that cGAS is responsible for the length discrimination, which may have mechanistic implications, but is not explored further in terms of DNA binding or cGAS activation, and the evidence provided here is somewhat preliminary.

Comments:

1.) The authors use one set of DNA fragments (PCR amplicons from a plasmid backbone) to draw their conclusions. Given that some instances of sequence-dependent recognition by cGAS have been reported (e.g. Herzner et al., 2016), it would have been important to show that any effects are independent of sequence by using several unrelated DNA fragments of similar lengths. A gel or similar showing visually that the DNA fragments are used at identical concentrations would have been a nice addition to EV1. Shorter DNA oligonucleotides (20mer) should also be tested, in case they can be detected at even higher concentrations.

2.) It should be confirmed that the lack of length-dependence at higher DNA concentrations in Fig. 1A is not due to saturation of the IFN bio-assay.

3.) The authors claim that there are two qualitatively different modes of cGAS-dependent DNA sensing, depending on the DNA concentration. For this, it would be important to show high and low DNA concentrations side by side in every experiment, not only in Fig. 1A. Is this the same in different cell types? Is this also true for other outputs (e.g. cytokine and chemokine mRNA and ELISA)?

4.) The STING- and cGAS depdence of DNA sensing in Fig 2 is hardly surpising. However, the observed STING-dependence of IL-1b production (EV 2C) is unexpected (should depend on AIM2 and ASC, not STING) - and this casts doubts over the ko cells used. There should be control experiments showing that STING and cGAS ko can still respond to other stimuli side by side.

5.) The length-dependence of P-TBK1 and STING dimerisation (Fig 2c, d) is not convincing, the lower exposure of the STING blot is unnecessary.

6.) cGAMP production shown in Fig. 2E and Fig, 3D-E are the key experiments in this manuscript. However, the authors should show cGAMP production for different DNA concentrations side by side, to confirm that length-dependence is abolished at higher DNA concentrations at the level of cGAS function, rather than it just being a function of a saturable IFNb production pathway.

7.) The authors speculate that the length recognition by cGAS argues against a model where cGAS binds dsDNA ends as a dimer, but do not provide any attempts to examine this further. The authors do not test the binding of cGAS to DNA fragments of different lengths (and at different concentrations) to separate cGAS DNA binding from function, or use different DNA species (circular, or with modified ends etc) to test this any further. It is possible that at low concentrations cGAS dimers bind preferentially to both ends of one dsDNA molecule, and longer fragments may be more flexible to allow for this kind of binding than an 88mer for instance. While interesting, the data presented here do not go far enough refute a model supported by previous structural and functional studies.

Referee #3:

In this work, Lueke et al. investigate the length-dependent activation of cGAS by DNA. Although previously thought not to matter for DNA > than ~45 nt , the authors made the interesting observation that DNA length was important for cGAS-STING activation by low dose of DNA (which is more physiological than that used in previous studies). The authors demonstrate that the dose-dependent effect of DNA length was visible in various human cells (primary and immortalised), and clearly showed involvement of cGAS and STING (using CRISPR KO cells). They also confirmed that cGAMP levels were directly proportional to DNA length (at low dose). The authors confirmed these observations in vitro using recombinant cGAS. Collectively, this elegant study has important implications in our understanding of cGAS function, and highlights the need for more mechanistic studies to define the mechanism underpinning the capacity of cGAS to preferentially detect longer DNA substrate (which is most likely important in the capacity of the cell to distinguish self and non-self DNA, as pointed out by the authors).

Specific comments to address:

1) The study relies on DNA fragments amplified from a pCDNA3.1 vector. Given that such vector contains bacterial specific genes, longer amplicons may contain sequences which could potentially interfere with the length phenotype (it is possible that certain sequences amplified are important too at low dose - i.e. that the previous report about sequence dependence not being important may also be biased by high concentrations - this is certainly the case with RNA sensing by TLR7/8 for instance). A control experiment comparing long and short DNA with, ideally, similar sequences (for instance repeats of the same sequence, where just the number of repeats vary between long an short DNA - non-bacterial), would help strengthen the overall claim of the paper.

2) the authors previously reported (Nat Immunol. 2012 Jun 17;13(8):737-43. doi: 10.1038/ni.2350.) that liposomes (prepared with Lipofectamine) can directly activate STING. As such, it is possible that the liposome used complex better with longer DNA to better activate STING. An experiment looking at electroporation of long and short DNAs should help resolve this point (using low dose electroporated DNA).

Minor points:

-Introduction section, page 3, line 46: the authors may also refer to the original paper rather than a review.

-Results section, page 6, line 84: the authors should discuss the activation of NFKB by STING for the reader to better understand the use of IL-1B.

1st Revision - authors' response 23 May 2017

Point-by-point response to the reviewers' comments:

Reviewer #1

Major concerns:

1. Choice of DNA ligands (applies to all experiments). The authors use PCR products of different lengths to investigate the length-dependency of the DNA-response. Using solely this technical approach poses several problems.

1.1. The authors use a silica-column-based purification approach to isolate PCR-products. This purification method can introduce a purity bias against smaller products. Usually, the amount of primers and dNTPs carried over is proportional to the amount of PCR reaction used, while the same volume of PCR reaction yields a higher mass of PCR product for higher lengths. This means that the same mass of a smaller PCR product will most likely have a higher contamination with primers and dNTPs, which cannot be distinguished in standard photometric quantification. The authors should carefully assess this by appropriate quantification methods (e.g. PicoGreen) and more importantly using other DNA sources, like synthetic oligonucleotides and plasmid DNA fragments (see also 1.3).

While the different degrees of contamination of PCR-derived DNA with primers and dNTPs is a valid concern, we chose to use the Nanodrop for determination of DNA concentration as there are reports that fluorescence-based assays tend to underestimate the DNA concentration of shorter DNA fragments compared to longer DNA (for example [1]). We'd like to point out that the high-sensitivity automated gel electrophoresis of the PCR fragments, which uses a DNA intercalating dye and fluorescence-based detection, does not show any contamination with primers (Fig. EV1A). However, this, of course, does not exclude the presence of amounts of contamination not detectable with this method. Therefore, we hope that this concern about contaminated PCR products is addressed to the reviewer's satisfaction by the use of two sets of DNA derived from restriction-digested plasmid added in the revised manuscript as suggested (Fig. 1F and 1G, Fig. 3F, please see response to comment 1.3).

1.2. Only one PCR-template is used. While this is a sound approach for an initial test, the resulting PCR-products may strongly vary in local and overall GC-content as well as PCR-efficiency and thereby purity (see 1.1.). The authors should at least include 2 representative additional PCR-products for each length, preferably with low and high GC content each, to assess the general applicability of the observed effects.

We have not included more PCR-derived sequences, but have instead included two sets of DNA derived from restriction digested plasmid, with a range of sequences and GC-contents (Fig. 1F and 1G, Fig. 3F, see also response to comment 1.3). We believe that the results obtained with these restriction fragments, which activate the IFN response and cGAS in a similarly length-dependent manner as the PCR-derived DNA, sufficiently rules out artefacts due to sequence content or purity.

1.3. Only PCR-products are used. The short PCR-products should be complemented with annealed synthetic oligonucleotides of defined length, also to address the concerns named in 1.1. Longer fragments should be added in the form of linearized as well as circular plasmids, which are readily available in the range of 3 to 20 kb in most laboratories. Plasmids digested with 4-mer cutters could also serve as a more reliable source of mixed fragments between 100 and 500 bp.

The authors furthermore argue that the observed effect may be important for the recognition of for instance the HSV-1 genome, which is very large. DNA of comparative size (approx. 150 kb) is also easily obtainable from bacterial artificial chromosomes, and thus should be included in the analyses.

We have now generated two sets of DNA by restriction digestion of the pOET1-OAS3 plasmid, one set derived from the vector backbone, ranging from 108 bp to 4570 bp and one set derived from the human OAS3 insert, ranging from 196 bp to 3317 bp (Fig. EV2A-C). These cover a range of sequences and GC-contents and do not elicit purity concerns as the PCR products do (see also response to comment 1.1 and 1.2). We have used both sets for IFN induction experiments in cell culture (Fig. 1F and 1G) and the vector backbone-derived one in an in vitro cGAS activity assay (Fig. 3F). They show the same length-dependency of IFN induction/cGAS activation. Please note that these restriction fragments stimulated the IFN response more strongly than the PCR-based fragments and we thus chose to reduce the concentration to 0.033 µg/ml (from 0.167 µg/ml) for the transfection experiments to avoid a saturation of the cGAS-STING pathway. We chose not to use annealed synthetic oligonucleotides here, because the focus of this study was the length dependency above 100 bp and the limit of commercially available synthetic oligonucleotides is app. 200 bp.

In the revised manuscript, we have also included data from an experiment where circular plasmid DNA was used in the in vitro cGAS activity assay (Fig. EV4E). The results show that circular DNA is able to induce cGAS activity despite a lack of DNA ends. These data demonstrate that cGAS-mediated DNA sensing does not rely on free DNA ends, as speculated previously [2,3].

Unfortunately, we have neither large plasmids in the range of up to 20 kb nor BACs available in our laboratory and we therefore didn't test longer lengths of DNA. We do agree that investigating even longer DNA lengths than the 4 kb investigated here would be relevant and might allow lowering the concentration used for stimulation further and possibly identifying the limits of length dependency. However, the main conclusion of this study, namely that cGAS activation remains length dependent many kilobases above the minimal stimulatory length, is solid without this data. We have softened the statement that the length-dependency of DNA recognition by cGAS is responsible for rapid detection of long pathogenic genomes in the discussion section (lines 158/159).

2. Choice of delivery method (applies to all experiments involving transfection)

2.1. Lipofectamine 2000 is used throughout the manuscript. All transfection reagents have a unique size bias depending on their mode of action and chemical properties. The authors should confirm their results with other reagents of different classes or generations (PEI, GeneJuice, Lipofectamine 3000). Reagents designed for small fragments like Lipofectamine RNAimax would be especially recommendable.

This is a very valid point. We have now transfected THP-1 cells with 0.167 µg/ml PCR-derived DNA using LipofectamineLTX (94 bp, 500 bp, and 4003 bp) (Fig. 1E) and LipofectamineRNAiMAX (94 bp vs 4003 bp) (Fig. EV1E) as requested. The length-dependent IFN induction (measured by type I IFN bioassay) can be observed with these delivery methods.
We've also tested IFN induction by DNA transfected by PEI-mediated delivery. However, the IFN induction we see with PEI was very low, even when increasing the incubation time from 12 h to 24 h. We did observe the same trend of length dependency, but the IFN response lacked robustness and consistency; therefore we do not feel comfortable including these data in the paper.

2.2. The transfection efficiency determination in Fig. 3A does not distinguish cGAS-available free cytosolic DNA from membrane-bound or intracellular intact Lipofectamine-DNA-particles. In fact, free DNA would have a very short half-life in the cytosol due to TREX1-mediated degradation. Therefore, a higher signal for the shorter PCR product could even suggest a delivery disadvantage for shorter DNA.

While we cannot formally exclude differences between the DNA lengths in efficiency of release from liposome-DNA particle into the cytosol after cell entry with this assay, we have not been able to find any indication of short DNA being delivered less efficiently than long DNA by lipofection. Also, the differences in transfection efficiency between the 94 bp and 4003 bp DNA are very small both at 30 min and 3 h after transfection.

We'd like to point out that, although we cannot fully exclude an effect of the delivery method and efficiency in the length dependency of IFN production observed in cellulo, the in vitro cGAS activity assays mirror the cell culture results very closely. Thus, differential delivery of the different length of DNA is highly unlikely to cause the observed length dependency (line 117/118).

2.3. The authors should use electroporation as a relatively unbiased and less "sticky" delivery method.

As requested, we attempted DNA delivery by electroporation to THP1 cells using the Nucleofector (Lonza). Unfortunately, the electroporated, PCR-derived dsDNA did not induce type I IFN production, neither at 12 h nor at 24 h post delivery (measured by type I IFN bioassay), not even when using the 4003 bp (long) dsDNA with a DNA amount/well corresponding to the high concentration in the transfection experiments (1.67 µg/ml 500 ng/well). This lack of IFN induction was seen despite successful delivery of a GFP-expression plasmid in parallel as confirmed by fluorescence microscopy at 24 h post delivery, which had an electroporation efficiency of app. 35 – 40 % and a cell viability of app. 45 – 50 %. Although this lack of stimulation by electroporated dsDNA in THP-1 cells is very interesting, we cannot explain it at the moment and find that further exploration of this issue would be beyond the scope of this study.

3. Role of TREX1 and general strength of the observed effects (applies to Fig. 3 B in comparison to other stimulations)

In most datasets, the authors use a small PCR product set of 94, 500 and 4003bp. In their laboratory THP-1 cell lines, a robust difference between the three PCR-products can be observed. Using TREX1-deficient THP-1 cells and respective control cells from Invivogen the authors claim that the observed differences are independent of TREX1. However, the difference between 500 bp and 4003 bp is barely visible in either TREX1-/- or WT cells, and also the response to the 94 bp fragment is only reduced by about 50 % in comparison to the 500 and 4003 bp fragments. The figure in its current form does not convincingly substantiate the claim that TREX1 is dispensable for the observed difference.

We agree with the reviewer that at the concentration used in the initial manuscript (0.167 µg/ml) for the experiments with TREX1 KO the length-dependency is not as pronounced in these THP-1 Dual cells as in the cells used for the other experiments. This is due to the fact that these cells (both the Dual control and the Dual TREX1 KO) are stimulated by DNA much more efficiently than our ATCC THP-1 cell line. Due to the higher stimulatory potential, the 0.167 µg/ml DNA is likely to already reach saturation of the cGAS-STING pathway, resulting in less obvious differences between the different DNA lengths. Therefore we have replaced the experiments with the TREX1 KO cells (both the IFN bioassay and the qPCR) with experiments using only 0.033 µg/ml (Fig 3B and EV4A). Those data now show clear length-dependency, both in control cells and in TREX1 KO cells, with the TREX1 KO showing higher overall levels of IFN production (including higher background IFN production).

4. cGAS in vitro assay (applies to Figure 3 D, E and EV3 C)

4.1. The general concern about the purity and accurate quantification of short PCR-products (1.1) applies here especially. The short PCR product will very likely have a higher contamination with primers and dNTPs than the longer ones. Apart from using a lower PCR-product concentration than intended and measured, primers might interfere with the reaction by non-productive binding of cGAS. Also, dATP from dNTPs can inefficiently be used as cGAS substrate in vitro (Gao et al., 2013), possibly leading to inhibition of cGAMP formation by competition. The authors should definitely repeat these experiments with defined fully synthetic short ligands replacing the 94 bp PCR-product and use linearized plasmid instead of or in addition to the 4003 bp PCR.

We have now repeated the in vitro cGAS activity assay using restriction fragments from a vector backbone of 108 bp, 568 bp, and 4570 bp. We chose not to include synthetic DNA for the short DNA length to allow for a more direct comparison with the longer, plasmid-derived ones. These restriction fragments activate cGAS in a similarly length-dependent manner as the PCR-derived fragments (Fig 3F, see also response to comment 1.3).

4.2. In EV3 C, the authors highlight "degradation products". What does this refer to? What is degraded? The lowest peaks for degradation products coincide with highest apparent cGAMP production. How is this related?

Please excuse the insufficient explanation of these in the initial manuscript. These degradation products are mainly ADP and GDP from the ATP and GTP substrates added to the reaction. This occurs since the stocks are not entirely clean of phosphatases. We have now included separate control elutions of cGAMP, ATP and GTP stocks, showing the degradation products in the ATP and GTP stocks (Fig. EV4E). In order to avoid substrate (ATP and GTP) depletion and reaction saturation to be able to show the length-dependency of cGAMP production, we chose to use very low DNA concentrations for the assay (1 ng/µl) and an excess of substrates. Therefore, the ATP and GTP and the corresponding ADP and GDP peaks are large compared to the cGAMP peaks. Nonetheless, the cGAMP production is detectable and reproducible in our assay setup. Therefore, we do not believe the degradation products are a cause for concern.

Reviewer #2

1.) The authors use one set of DNA fragments (PCR amplicons from a plasmid backbone) to draw their conclusions. Given that some instances of sequence-dependent recognition by cGAS have been reported (e.g. Herzner et al., 2016), it would have been important to show that any effects are independent of sequence by using several unrelated DNA fragments of similar lengths. A gel or similar showing visually that the DNA fragments are used at identical concentrations would have been a nice addition to EV1. Shorter DNA oligonucleotides (20mer) should also be tested, in case they can be detected at even higher concentrations.

We have now included two sets of restriction fragments generated from the pOET1-OAS3 plasmid, one set derived from the plasmid backbone and one set derived from the human OAS3 insert. These cover a range of sequences and GC-contents and induce the IFN response in a similarly length-dependent manner as the PCR products (Fig. 1F and 1G, please refer to response to reviewer #1, comment 1.3 for more detail). As requested, we've now included agarose gels of the DNA stocks used for transfection of cells to control for the concentrations (Fig EV1B, EV2C). Due to different "sharpness" of the bands, the intensities vary slightly between the bands, but it is clear that the longer DNA species were not used at higher concentrations. We chose not to use short synthetic oligonucleotides here, because the focus of this study was the length dependency above 100 bp. The length-dependency near the minimal stimulatory unit (<50 bp) for IFN response induced by cytosolic DNA has been investigated thoroughly previously [4].

2.) It should be confirmed that the lack of length-dependence at higher DNA concentrations in Fig. 1A is not due to saturation of the IFN bio-assay.

The authors are fully aware of this potential pitfall, and have therefore taken a dual approach, with IFN bioassay and qPCR as readouts. The data shown in Fig. 1A was seen in several independent experiments using different dilutions of the supernatants. Moreover, the measurement of IFNβ mRNA levels by qPCR (Fig. EV1C), lead to data well within the dynamic range of this assay (Ct ranging from app. 28 to 20) which show the same lack of length-dependency of the IFNβ response when transfecting with the high concentration. Thus, the authors are convinced that the lack of length-dependence at higher DNA concentrations in Fig. 1A is not due to saturation of the IFN bio-assay.

3.) The authors claim that there are two qualitatively different modes of cGAS-dependent DNA sensing, depending on the DNA concentration. For this, it would be important to show high and low DNA concentrations side by side in every experiment, not only in Fig. 1A. Is this the same in different cell types? Is this also true for other outputs (e.g. cytokine and chemokine mRNA and ELISA)?

We apologize if the impression has arisen that we hypothesize the presence of two qualitatively different modes of cGAS activation, one for low and one for high concentration. Actually, we think that cGAS activation is always length-dependent, independent of the DNA concentration (given sufficient substrate supply), but that this length-dependency only becomes visible on the level of IFN production at low DNA concentration, as high concentration quickly leads to a saturation of the

IFN induction pathway. To make this clearer in the manuscript, we have now included experiments showing length-dependent cGAMP production, STING dimerization, and TBK1 phoshorylation after transfection of cells with a high DNA concentration (1.67 µg/ml) (Fig. EV3D-F), indicating that the saturation of the IFN pathway at high DNA concentrations occurs downstream of TBK1 phosphorylation.

4.) The STING- and cGAS depdence of DNA sensing in Fig 2 is hardly surprising. However, the observed STING-dependence of IL-1b production (EV 2C) is unexpected (should depend on AIM2 and ASC, not STING) - and this casts doubts over the ko cells used. There should be control experiments showing that STING and cGAS ko can still respond to other stimuli side by side.

The data presented on Fig. EV2C in the original manuscript could be explained by DNA-mediated activation of STING-dependent pro-IL-1β.We agree that this has not been sufficiently commented on and investigated in the manuscript. However, since this study has focused even more on the the cGAS-STING-IFN axis during revision, the authors now find that the IL-1β data are not well placed in this paper. Therefore we've decided to remove the IL-1β data (Fig. 1C and EV2C in the previous version) to allow for use in later publications and to keep a better focus in this manuscript.
Nontheless, the request for validation of the knockout cell lines used here is a valid point. For the control stimulation experiments, we'd like to refer to the studies in which these knockout cells were first published [5,6]. We've cited these in the manuscript. However, we've now included current control Western blots of the knockout cell lines used showing cGAS, STING, IFI16 and TREX1 levels in all cell lines (Fig. EV3A).

5.) The length-dependence of P-TBK1 and STING dimerisation (Fig 2c, d) is not convincing, the lower exposure of the STING blot is unnecessary.

On this point, we must respectfully disagree with reviewer #2. Fig. 2C, top panel, is a clear blot showing p-TBK1 absent in mock transfected cells, present in a small amount in cells transfected with 94 bp DNA at 0.167 µg/ml and present in a larger amount in cells transfected with 4003 bp DNA at 0.167 µg/ml. The corresponding blots for total TBK1 and for the loading control vinculin show equal levels of these proteins across all samples. Similarly, the STING dimer is present at very low levels in mock transfected cells and its level increases with the transfected DNA length (best visible in the high exposure), while the STING monomer decreases with increasing DNA length (best visible in the low exposure), likely representing both migration of STING to the dimerized form and degradation of STING. The vinculin loading control again shows equal protein levels across all samples. We've chosen to show both the high and low exposure of the blot to allow for easy visualization of both the dimerized and monomeric form of STING.

6.) cGAMP production shown in Fig. 2E and Fig, 3D-E are the key experiments in this manuscript. However, the authors should show cGAMP production for different DNA concentrations side by side, to confirm that length-dependence is abolished at higher DNA concentrations at the level of cGAS function, rather than it just being a function of a saturable IFNb production pathway.

As mentioned in response to comment 3, we did not intend to imply in any way that the length-dependency would disappear at high concentrations on the level of cGAMP production, but rather think that the length-dependency becomes "invisible" on the level of IFN readout at high DNA concentrations due to a saturation of the IFN inducing pathway. We've now included mass spectrometry quantification of cGAMP levels in cells transfected with a high concentration of DNA (1.67 µg/ml) of 94 bp or 4003 bp (Fig. EV3F). The results show that cGAMP production is length-dependent also at this high concentration.

7.) The authors speculate that the length recognition by cGAS argues against a model where cGAS binds dsDNA ends as a dimer, but do not provide any attempts to examine this further. The authors do not test the binding of cGAS to DNA fragments of different lengths (and at different concentrations) to separate cGAS DNA binding from function, or use different DNA species (circular, or with modified ends etc) to test this any further. It is possible that at low concentrations cGAS dimers bind preferentially to both ends of one dsDNA molecule, and longer fragments may be more flexible to allow for this kind of binding than an 88mer for instance. While interesting, the data presented here do not go far enough refute a model supported by previous structural and functional studies.

To clarify, we speculate only that the DNA ends may not play such an essential role in cGAS activation as stipulated previously; however we do not argue against cGAS binding DNA as a dimer. We have considered the model of a cGAS dimer binding both ends of long DNA molecule preferentially over binding two separate short DNA molecules. While we agree that this is an attractive model to explain the difference in activity induced e.g. by 94 bp DNA vs a 500 bp DNA, this is unlikely to explain the increased activity induced by a 4003 bp long DNA molecule due to its size. We've now added data showing that a circular (undigested) plasmid DNA can activate cGAMP production in vitro very well (Fig. EV4F); however, a direct comparison of circular and linear plasmids is difficult due to concerns about different supercoiled populations in the circular plasmid preparations. Nonetheless, we interpret this data together with the length-dependency to argue against a mandatory requirement for DNA ends for cGAS activation. We'd like to note that this is not a major point of the study and that our discussion of the matter is phrased carefully ("It should be noted that these data argue against…" lines 172/173). Although a more thorough investigation of the issue of requirement for DNA ends and further mechanistic studies are beyond the scope of this study, we believe the data presented here warrant the inclusion of this point in the manuscript in order to re-initiate a discussion of this important issue in the field.

Reviewer #3:

1) The study relies on DNA fragments amplified from a pCDNA3.1 vector. Given that such vector contains bacterial specific genes, longer amplicons may contain sequences which could potentially interfere with the length phenotype (it is possible that certain sequences amplified are important too at low dose - i.e. that the previous report about sequence dependence not being important may also be biased by high concentrations - this is certainly the case with RNA sensing by TLR7/8 for instance). A control experiment comparing long and short DNA with, ideally, similar sequences (for instance repeats of the same sequence, where just the number of repeats vary between long an short DNA - non-bacterial), would help strengthen the overall claim of the paper.

The request for proof of sequence independency of the observed effect is very valid. Although we did not generate short and long DNA species with repeats of the same sequences, we have now included two sets of DNA by restriction digestion of the pOET1-OAS3 plasmid, one set derived from the vector backbone and one set derived from the human OAS3 insert (Fig. EV2 A-C). These cover a range of sequences and show similar length dependency as the PCR-generated fragments (Fig. 1F, 1G and 3F). Please also see response to reviewer #1, comment 1.3 for further details. The authors hope that the inclusion of sequences from the human gene OAS3 (as an insert in the pOET1-OAS3 vector) will resolve the reviewer's concern that bacteria-specific sequences/genes have an influence in this context.

2) The authors previously reported (Nat Immunol. 2012 Jun 17;13(8):737-43. doi: 10.1038/ni.2350.) that liposomes (prepared with Lipofectamine) can directly activate STING. As such, it is possible that the liposome used complex better with longer DNA to better activate STING. An experiment looking at electroporation of long and short DNAs should help resolve this point (using low dose electroporated DNA).

We agree that delivery of dsDNA to THP1 cells by electroporation would have been a very nice addition to the study. However, our attempts to elicit an IFN response by electroporated DNA in THP-1 were not successful (for details, please refer to response to reviewer #1, comment 2.3). Other lipofection-based delivery methods, including LipofectamineRNAiMAX, which is optimized for delivery of short nucleic acids, resulted in length-dependent stimulation of the IFN response (Fig. 1E and EV1E, for details please see to response to reviewer #1, comment 2.1). As mentioned above, while we cannot fully exclude an effect of the delivery method and efficiency in the length dependency, the in vitro cGAS activity assays mirror the cell culture results very closely. Thus, the delivery of the different length of DNA is highly unlikely to cause the observed length dependency.

Minor points:

-Introduction section, page 3, line 46: the authors may also refer to the original paper rather than a review.

This has been rectified. We now cite [7] for the sequence independency of cGAS-DNA binding and [8] for structural evidence of a preference for B-form DNA.

-Results section, page 6, line 84: the authors should discuss the activation of NFKB by STING for the reader to better understand the use of IL-1B.

We have removed the results with IL-1β (please see response to reviewer #2, comment 4).

References referred to in point-by-point response

1. Sedlackova T, Repiska G, Celec P, Szemes T, Minarik G (2013) Fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods. *Biological procedures online* **15:** 5
2. Li X, Shu C, Yi G, Chaton CT, Shelton CL, Diao J, Zuo X, Kao CC, Herr AB, Li P (2013) Cyclic GMP-AMP synthase is activated by double-stranded DNA-induced oligomerization. *Immunity* **39:** 1019-1031
3. Kranzusch PJ, Vance RE (2013) cGAS dimerization entangles DNA recognition. *Immunity* **39:** 992-994
4. Karayel E, Burckstummer T, Bilban M, Durnberger G, Weitzer S, Martinez J, Superti-Furga G (2009) The TLR-independent DNA recognition pathway in murine macrophages: Ligand features and molecular signature. *European journal of immunology* **39:** 1929-1936
5. Holm CK, Rahbek SH, Gad HH, Bak RO, Jakobsen MR, Jiang Z, Hansen AL, Jensen SK, Sun C, Thomsen MK*, et al.* (2016) Influenza A virus targets a cGAS-independent STING pathway that controls enveloped RNA viruses. *Nature communications* **7:** 10680
6. Jonsson KL, Laustsen A, Krapp C, Skipper KA, Thavachelvam K, Hotter D, Egedal JH, Kjolby M, Mohammadi P, Prabakaran T*, et al.* (2017) IFI16 is required for DNA sensing in human macrophages by promoting production and function of cGAMP. *Nature communications* **8:** 14391
7. Sun L, Wu J, Du F, Chen X, Chen ZJ (2013) Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science (New York, N.Y.)* **339:** 786-791
8. Civril F, Deimling T, de Oliveira Mann CC, Ablasser A, Moldt M, Witte G, Hornung V, Hopfner KP (2013) Structural mechanism of cytosolic DNA sensing by cGAS. *Nature* **498:** 332-337

2nd Editorial Decision 16 June 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study (you will find enclosed below). Referee #3 now supports the publication of your study in EMBO reports, whereas referees #1 and #2 have still some concerns, we ask you to address in a final revised version of your manuscript. I also have two requests that I ask you to address during revision:

I would suggest to change the title into something more active. E.g.: Cytosolic DNA sensing by the cyclic GMP-AMP synthase cGAS is length-dependent.

The graphs in Figures EV1A and EV2 A-B have rather small numbers that are hard to read, and the far right values are overlapping and can't be read at all. Please provide these graphs with bigger fonts and non-overlapping numbers.

REFEREE REPORTS

Referee #1:

The authors have only rather superficially addressed the concerns raised by this and other reviewers and avoided important experiments that were deemed necessary to substantiate the presented claims. Most importantly, the authors do not confirm their results with any DNA delivery method besides lipofection. This concern is critical as different length-dependent behavior of transfected DNA could be solely a technical effect of the delivery stoichiometry. The authors' central argument against this possibility is the in vitro cGAS activation assay. However, still lacking here is the straight forward and most definitive control of using synthetic dsDNA of identical sequence to the PCR products, which the authors deliberately avoid despite our suggestion. Moreover, the new data using DNA

restriction fragments show only a minimal difference between the 4 kb and 500 bp fragments. Additional comments are outlined below in response to the point-to-point reply. In its current form, the manuscript although improved does not provide sufficient evidence of the authors' claims and cannot be considered without further substantial revision.

Point-by-point response (authors' original responses in italics, reviewer's responses in bold):

Major concerns:

1. Choice of DNA ligands (applies to all experiments). The authors use PCR products of different lengths to investigate the length-dependency of the DNA-response. Using solely this technical approach poses several problems.

1.1. The authors use a silica-column-based purification approach to isolate PCR-products. This purification method can introduce a purity bias against smaller products. Usually, the amount of primers and dNTPs carried over is proportional to the amount of PCR reaction used, while the same volume of PCR reaction yields a higher mass of PCR product for higher lengths. This means that the same mass of a smaller PCR product will most likely have a higher contamination with primers and dNTPs, which cannot be distinguished in standard photometric quantification. The authors should carefully assess this by appropriate quantification methods (e.g. PicoGreen) and more importantly using other DNA sources, like synthetic oligonucleotides and plasmid DNA fragments (see also 1.3).

While the different degrees of contamination of PCR-derived DNA with primers and dNTPs is a valid concern, we chose to use the Nanodrop for determination of DNA concentration as there are reports that fluorescence-based assays tend to underestimate the DNA concentration of shorter DNA fragments compared to longer DNA (for example [1]).

We'd like to point out that the high-sensitivity automated gel electrophoresis of the PCR fragments, which uses a DNA intercalating dye and fluorescence-based detection, does not show any contamination with primers (Fig. EV1A). However, this, of course, does not exclude the presence of amounts of contamination not detectable with this method.

Therefore, we hope that this concern about contaminated PCR products is addressed to the reviewer's satisfaction by the use of two sets of DNA derived from restriction-digested plasmid added in the revised manuscript as suggested (Fig. 1F and 1G, Fig. 3F, please see response to comment 1.3).

R1: While different methods of quantification certainly have their individual advantages and disadvantages, spectrophotometric quantification is particularly vulnerable to contamination with dNTPs, which is a major concern for PCR products. Even if, as implied by the authors' response, picogreen (https://www.thermofisher.com/order/catalog/product/P11496) measurement would potentially underestimate the concentration of small fragments, the error would only be in favor of the authors' hypothesis. Therefore confirming the DNA concentrations using this or another dsDNA-specific method remains an issue. Moreover, as suggested previously, annealed synthetic DNA oligonucleotides of identical sequence as the short PCR products and restriction fragments are very important controls for both reliability of quantitation and stimulatory properties that should be included (see point 1.3)

1.2. Only one PCR-template is used. While this is a sound approach for an initial test, the resulting PCR-products may strongly vary in local and overall GC-content as well as PCR-efficiency and thereby purity (see 1.1.). The authors should at least include 2 representative additional PCR-products for each length, preferably with low and high GC content each, to assess the general applicability of the observed effects.

We have not included more PCR-derived sequences, but have instead included two sets of DNA derived from restriction digested plasmid, with a range of sequences and GC-contents (Fig. 1F and 1G, Fig. 3F, see also response to comment 1.3). We believe that the results obtained with these restriction fragments, which activate the IFN response and cGAS in a similarly length-dependent manner as the PCR-derived DNA, sufficiently rules out artefacts due to sequence content or purity.

1.3. Only PCR-products are used. The short PCR-products should be complemented with annealed synthetic oligonucleotides of defined length, also to address the concerns named in 1.1. Longer fragments should be added in the form of linearized as well as circular plasmids, which are readily

available in the range of 3 to 20 kb in most laboratories. Plasmids digested with 4-mer cutters could also serve as a more reliable source of mixed fragments between 100 and 500 bp.

The authors furthermore argue that the observed effect may be important for the recognition of for instance the HSV-1 genome, which is very large. DNA of comparative size (approx. 150 kb) is also easily obtainable from bacterial artificial chromosomes, and thus should be included in the analyses. We have now generated two sets of DNA by restriction digestion of the pOET1-OAS3 plasmid, one set derived from the vector backbone, ranging from 108 bp to 4570 bp and one set derived from the human OAS3 insert, ranging from 196 bp to 3317 bp (Fig. EV2A-C). These cover a range of sequences and GC-contents and do not elicit purity concerns as the PCR products do (see also response to comment 1.1 and 1.2). We have used both sets for IFN induction experiments in cell culture (Fig. 1F and 1G) and the vector backbone-derived one in an in vitro cGAS activity assay (Fig. 3F). They show the same length-dependency of IFN induction/cGAS activation. Please note that these restriction fragments stimulated the IFN response more strongly than the PCR-based fragments and we thus chose to reduce the concentration to 0.033 µg/ml (from 0.167 µg/ml) for the transfection experiments to avoid a saturation of the cGAS-STING pathway.

We chose not to use annealed synthetic oligonucleotides here, because the focus of this study was the length dependency above 100 bp and the limit of commercially available synthetic oligonucleotides is app. 200 bp.

R1: The main purpose of synthetic oligonucleotides here would be to serve as a control for both accuracy of quantitation and activity in direct comparison to short PCR products and restriction fragments of identical sequence and therefore should definitely be included.

In the revised manuscript, we have also included data from an experiment where circular plasmid DNA was used in the in vitro cGAS activity assay (Fig. EV4E). The results show that circular DNA is able to induce cGAS activity despite a lack of DNA ends. These data demonstrate that cGAS-mediated DNA sensing does not rely on free DNA ends, as speculated previously [2,3].

Unfortunately, we have neither large plasmids in the range of up to 20 kb nor BACs available in our laboratory and we therefore didn't test longer lengths of DNA. We do agree that investigating even longer DNA lengths than the 4 kb investigated here would be relevant and might allow lowering the concentration used for stimulation further and possibly identifying the limits of length dependency.

However, the main conclusion of this study, namely that cGAS activation remains length dependent many kilobases above the minimal stimulatory length, is solid without this data. We have softened the statement that the length-dependency of DNA recognition by cGAS is responsible for rapid detection of long pathogenic genomes in the discussion section (lines 158/159).

R1: Even if these DNAs should not be available in the authors' laboratory, BACs can readily be ordered from not-for-profit repositories or companies and large plasmids can be easily obtained from addgene or from other laboratories.

2. Choice of delivery method (applies to all experiments involving transfection)

2.1. Lipofectamine 2000 is used throughout the manuscript. All transfection reagents have a unique size bias depending on their mode of action and chemical properties. The authors should confirm their results with other reagents of different classes or generations (PEI, GeneJuice, Lipofectamine 3000). Reagents designed for small fragments like Lipofectamine RNAimax would be especially recommendable.

This is a very valid point. We have now transfected THP-1 cells with 0.167 µg/ml PCR-derived DNA using LipofectamineLTX (94 bp, 500 bp, and 4003 bp) (Fig. 1E) and Lipofectamine RNAiMAX (94 bp vs 4003 bp) (Fig. EV1E) as requested. The length-dependent IFN induction (measured by type I IFN bioassay) can be observed with these delivery methods. We've also tested IFN induction by DNA transfected by PEI-mediated delivery. However, the IFN induction we see with PEI was very low, even when increasing the incubation time from 12 h to 24 h. We did observe the same trend of length dependency, but the IFN response lacked robustness and consistency; therefore we do not feel comfortable including these data in the paper.

2.2. The transfection efficiency determination in Fig. 3A does not distinguish cGAS-available free cytosolic DNA from membrane-bound or intracellular intact Lipofectamine-DNA-particles. In fact, free DNA would have a very short half-life in the cytosol due to TREX1-mediated degradation.

Therefore, a higher signal for the shorter PCR product could even suggest a delivery disadvantage for shorter DNA.

While we cannot formally exclude differences between the DNA lengths in efficiency of release from liposome-DNA particle into the cytosol after cell entry with this assay, we have not been able to find any indication of short DNA being delivered less efficiently than long DNA by lipofection. Also, the differences in transfection efficiency between the 94 bp and 4003 bp DNA are very small both at 30 min and 3 h after transfection. We'd like to point out that, although we cannot fully exclude an effect of the delivery method and efficiency in the length dependency of IFN production observed in cellulo, the in vitro cGAS activity assays mirror the cell culture results very closely. Thus, differential delivery of the different length of DNA is highly unlikely to cause the observed length dependency (line 117/118).

2.3. The authors should use electroporation as a relatively unbiased and less "sticky" delivery method.

As requested, we attempted DNA delivery by electroporation to THP1 cells using the Nucleofector (Lonza). Unfortunately, the electroporated, PCR-derived dsDNA did not induce type I IFN production, neither at 12 h nor at 24 h post delivery (measured by type I IFN bioassay), not even when using the 4003 bp (long) dsDNA with a DNA amount/well corresponding to the high concentration in the transfection experiments (1.67 µg/ml 500 ng/well). This lack of IFN induction was seen despite successful delivery of a GFP-expression plasmid in parallel as confirmed by fluorescence microscopy at 24 h post delivery, which had an electroporation efficiency of app. 35 - 40 % and a cell viability of app. 45 - 50 %. Although this lack of stimulation by electroporated dsDNA in THP-1 cells is very interesting, we cannot explain it at the moment and find that further exploration of this issue would be beyond the scope of this study.

R1: Electroporation naturally requires higher DNA amounts than lipofection, as DNA is not targeted to the cell but instead evenly distributed throughout the electroporation suspension. At least the same amount as used for the GFP plasmid should be used. Has type I IFN or CXCL-10 been measured after plasmid electroporation?

The authors could alternatively apply other delivery methods like digitonin permeabilization. Many of the authors' findings could be partially or completely explained by transfection mechanistics. Here the burden of proof critically lies with the authors. Showing transfection reagent-independent delivery would greatly strengthen the manuscript.

3. Role of TREX1 and general strength of the observed effects (applies to Fig. 3 B in comparison to other stimulations)

In most datasets, the authors use a small PCR product set of 94, 500 and 4003bp. In their laboratory THP-1 cell lines, a robust difference between the three PCR-products can be observed. Using TREX1-deficient THP-1 cells and respective control cells from Invivogen the authors claim that the observed differences are independent of TREX1. However, the difference between 500 bp and 4003 bp is barely visible in either TREX1-/- or WT cells, and also the response to the 94 bp fragment is only reduced by about 50 % in comparison to the 500 and 4003 bp fragments. The figure in its current form does not convincingly substantiate the claim that TREX1 is dispensable for the observed difference.

We agree with the reviewer that at the concentration used in the initial manuscript (0.167 µg/ml) for the experiments with TREX1 KO the length-dependency is not as pronounced in these THP-1 Dual cells as in the cells used for the other experiments. This is due to the fact that these cells (both the Dual control and the Dual TREX1 KO) are stimulated by DNA much more efficiently than our ATCC THP-1 cell line. Due to the higher stimulatory potential, the 0.167 µg/ml DNA is likely to already reach saturation of the cGAS-STING pathway, resulting in less obvious differences between the different DNA lengths. Therefore we have replaced the experiments with the TREX1 KO cells (both the IFN bioassay and the qPCR) with experiments using only 0.033 µg/ml (Fig 3B and EV4A). Those data now show clear length-dependency, both in control cells and in TREX1 KO cells, with the TREX1 KO showing higher overall levels of IFN production (including higher background IFN production).

4. cGAS in vitro assay (applies to Figure 3 D, E and EV3 C)

4.1. The general concern about the purity and accurate quantification of short PCR-products (1.1) applies here especially. The short PCR product will very likely have a higher contamination with primers and dNTPs than the longer ones. Apart from using a lower PCR-product concentration than intended and measured, primers might interfere with the reaction by non-productive binding of cGAS. Also, dATP from dNTPs can inefficiently be used as cGAS substrate in vitro (Gao et al., 2013), possibly leading to inhibition of cGAMP formation by competition. The authors should definitely repeat these experiments with defined fully synthetic short ligands replacing the 94 bp PCR-product and use linearized plasmid instead of or in addition to the 4003 bp PCR.

We have now repeated the in vitro cGAS activity assay using restriction fragments from a vector backbone of 108 bp, 568 bp, and 4570 bp. We chose not to include synthetic DNA for the short DNA length to allow for a more direct comparison with the longer, plasmid-derived ones. These restriction fragments activate cGAS in a similarly length-dependent manner as the PCR-derived fragments (Fig 3F, see also response to comment 1.3).

R1: While the use of restriction fragments is an improvement, the restriction fragments of 568bp and 4570bp show very similar activity in the assay. The 108 bp fragment is rather weak as expected, which however could also be due to purification bias. A synthetic version of the 108bp fragment should be included in the assay.

4.2. In EV3 C, the authors highlight "degradation products". What does this refer to? What is degraded? The lowest peaks for degradation products coincide with highest apparent cGAMP production. How is this related?

Please excuse the insufficient explanation of these in the initial manuscript. These degradation products are mainly ADP and GDP from the ATP and GTP substrates added to the reaction. This occurs since the stocks are not entirely clean of phosphatases. We have now included separate control elutions of cGAMP, ATP and GTP stocks, showing the degradation products in the ATP and GTP stocks (Fig. EV4E). In order to avoid substrate (ATP and GTP) depletion and reaction saturation to be able to show the length-dependency of cGAMP production, we chose to use very low DNA concentrations for the assay (1 ng/µl) and an excess of substrates. Therefore, the ATP and GTP and the corresponding ADP and GDP peaks are large compared to the cGAMP peaks. Nonetheless, the cGAMP production is detectable and reproducible in our assay setup. Therefore, we do not believe the degradation products are a cause for concern.

Referee #2:

In the revised version of this manuscript the authors now shore more convincingly that DNA recognition by cGAS is length-dependent, having addressed the major pitfall of the previous version by using DNA fragments from different sources and different transfection methods. In my opinion, this work now provides an important novel piece of information on the mechanism of DNA sensing, which would provide the groundwork for further molecular and structural studies which will be required to explain this finding. The figures and data are generally well controlled and convincing, greatly benefiting from the additional evidence shown in this revised version.

Minor comments:

1.) The existence of other DNA co-receptors/co-factors may be introduced earlier (given that the function of IFI16 is examined in this context) - e.g. in the introduction and/or on p.6 after: "cGas has been established as the main IFN-inducing cytosolic DNA sensor".

2.) The band corresponding to phosphorylated STING should be highlighted in Fig. 2D, or lysates could be re-blotted with phospho-STING (Ser366) antibody which is available commercially.

3.) Can the authors speculate on the mechanism of saturation for the IFN response seen at higher DNA concentrations, which is proposed to occur downstream of TBK1 - i.e. have the authors looked at IRF3 phosphorylation or nuclear translocation?

4.) in Fig EV 2A, the blot for IFI16 ko cells shows lower cGAS levels, possibly explained by the lower levels of the loading control vinculin. This may be misleading, as it can hint towards an indirect role of IFI16 in DNA sensing via cGAS expression (which is unlikely to be true). An improved blot of wt and IFI16 ko cells with even loading control (and even cGAS levels, as shown

previously in these cells) is recommended.

Referee #3:

The authors have answered my concerns: the additional data with digestion products from two different regions of a plasmid (including a set based on a human gene), clearly establishes that the effect is not limited to that of PCR products, and is not limited to the bacterial sequences in the vector. The demonstration that all the transfection reagents testes also led to the same observation reinforces the concept that the contribution of this factor is likely negligible to the phenotype described - also seen in vitro with recombinant cGAS. The clarification that the length dependent effect is also visible at high dose, now included was also important. Altogether, the claims are now strongly supported by the data.

2nd Revision - authors' response 06 July 2017

Point-by-point response (responses in red)

Editor:

I also have the two requests that I ask you to address during revision.

I would suggest to change the title into something more active. E.g.: Cytosolic DNA sensing by the cyclic GMP-AMP synthase cGAS is length-dependent

The title has been changed to "*cGAS is activated by DNA in a length-dependent manner*"

The graphs in Figures EV1A and EV2 A-B have rather small numbers that are hard to read, and the far right values are overlapping and can't be read at all. Please provide these graphs with bigger fonts and non-overlapping numbers.

The graphs taken directly from the Fragment Analyzer analysis software (ProSize 2.0) have been replaced with graphs generated in GraphPad Prism 7. Font is now Arial, size 8 pt.

Referee #1:

The authors have only rather superficially addressed the concerns raised by this and other reviewers and avoided important experiments that were deemed necessary to substantiate the presented claims. Most importantly, the authors do not confirm their results with any DNA delivery method besides lipofection. This concern is critical as different length-dependent behavior of transfected DNA could be solely a technical effect of the delivery stoichiometry. The authors' central argument against this possibility is the in vitro cGAS activation assay. However, still lacking here is the straight forward and most definitive control of using synthetic dsDNA of identical sequence to the PCR products, which the authors deliberately avoid despite our suggestion. Moreover, the new data using DNA restriction fragments show only a minimal difference between the 4 kb and 500 bp fragments. Additional comments are outlined below in response to the point-to-point reply. In its current form, the manuscript although improved does not provide sufficient evidence of the authors' claims and cannot be considered without further substantial revision.

As we understand it, reviewer #1's criticism of our study now centers around 4 main concerns:

a) Mode of DNA delivery.

Reviewer #1 finds it a serious problem that all cell data are based on lipofection-based delivery. We would of course have preferred to provide strong data from experiments with a different delivery method, and did test Amaxa, PEI, and in fact also Digitonin permeabilization as suggested by reviewer #1. As already described in the first point-by-point response, these modes of delivery did not lead to strong IFN induction. Although this is a phenomenon that should be explained, the lipofection-based DNA delivery method is commonly used in the cGAS-STING field.

We would like to emphasize that the length-dependent cGAS activation is also seen in vitro, thus strongly arguing against our observations being an artifact caused by lipofection-mediated delivery. Moreover, among the lipofection-based methods we tested RNAiMax, which is optimized for delivery of small DNAs, and we still observed length-dependent IFN induction. Thus, the authors find it very unlikely that the results of the present work are explained by the mode of DNA delivery used.

b) A need for data from synthetic dsDNAs.

This point is mainly based on the fact that reviewer #1 is still not convinced that our dsDNAs are free of contaminating dNTPs. We find that a very unlikely possibility. In the revised manuscript we provided confirmatory data based on gel-purified restriction digestion fragments. It is very unlikely that such dsDNAs contain contaminating nucleotides to an extent that would influence the results, and explain our findings.

c) DNA quantification should be done with a fluorescence-based method, such as PicoGreen.

As requested, we have now used a PicoGreen assay to determine the DNA concentration in the working stocks of PCR products and restriction fragments used for cell transfection. Please see the table below (Table PBPR1) for a comparison of the NanoDrop and PicoGreen measurements.

As expected from literature (Sedlackova *et al.*, 2013), the PicoGreen measurement underestimates the amount of DNA for the short dsDNA. This occurs to a similar extent for the PCR products and for the restriction fragments. Therefore, we are confident that this is mainly due to the documented effect of PicoGreen underestimating the concentration of short DNA lengths and not due to contamination with dNTPs.

Nonetheless, to further dispel the reviewer's concern about the different possible methods to determine DNA concentration, we show below the type I IFN production from PMA-differentiated THP1 cells transfected with three PCR products of different lengths (0.167 µg/ml), where the transfected amount of DNA was calculated according to a PicoGreen-based concentration determination (Figure PBPR1). Also with this method of concentration determination, the longer the DNA lengths, the more type I IFN is induced. We have included the results of the measurements below, but do not feel it necessary to include them in the manuscript.

Table PBPR1: Concentration of DNA stocks used for cell transfection by Nanodrop and by PicoGreen

DNA origin	DNA size [bp]	Concentration measured by NanoDrop [ng/µl]	Concentration measured by PicoGreen assay [ng/µl]
PCR products	88	109	58
	94	110	62
	300	109	99
	500	112	106
	836	107	96
	2027	105	103
	4003	112	102
Restriction fragments (vector backbone-	108	33	20
	568	31	27

derived)	1383	32	23
	4570	32	29
Restriction fragments (OAS3 gene-derived)	196	30	17
	515	32	23
	826	28	24
	1777	33	27
	3317	30	24

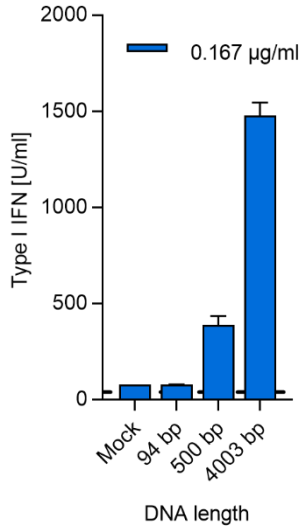

Figure PBPR1: Type I IFN levels in supernatants from PMA-differentiated THP-1 cells transfected with Lipofectamine2000 with PCR-derived dsDNA of indicated lengths at 0.167 µg/ml for 12 h, measured by bioassay. Concentration measurement of DNA stocks by PicoGreen assay was used to determine amount of DNA to transfect.

d) BAC-derived DNA (or other DNA of comparable length) should be tested.

In this manuscript we test DNAs up a length of 4500 bps and we demonstrate that the length dependency is observed over a very long length-range. While it could be interesting to test DNA species even longer (as could be done with BACs or isolated viral genomes), it is difficult for us to see how this would consolidate the main findings of the work or add anything conceptually to the conclusion. Also, while comparable delivery of the DNA lengths used in this study (app. 100 bp – 4500 bp) is easily achievable by transfection, reliable delivery of such long DNA (e.g. 150000 bp) in a manner that allows for direct comparison to shorter DNA would be difficult.

Referee #2:

In the revised version of this manuscript the authors now show more convincingly that DNA recognition by cGAS is length-dependent, having addressed the major pitfall of the previous version by using DNA fragments from different sources and different transfection methods. In my opinion, this work now provides an important novel piece of information on the mechanism of DNA sensing, which would provide the groundwork for further molecular and structural studies which will be required to explain this finding. The figures and data are generally well controlled and convincing, greatly benefiting from the additional evidence shown in this revised version.

Minor comments:

1.) The existence of other DNA co-receptors/co-factors may be introduced earlier (given that the function of IFI16 is examined in this context) - e.g. in the introduction and/or on p.6 after: "cGas has been established as the main IFN-inducing cytosolic DNA sensor."

The following sentence has now been added to the first paragraph of the introduction (p. 3): “Other intracellular proteins have been suggested to act as innate DNA receptors, such as IFI16, which is now thought to be a co-factor for STING-dependent DNA sensing in human cells”. We have not mentioned IFI16 on page 6 since this part of the results section focuses on cGAS.

2.) The band corresponding to phosphorylated STING should be highlighted in Fig. 2D, or lysates could be re-blotted with phospho-STING (Ser366) antibody which is available commercially.

The p-STING blot has been added (Fig 2D). As dimerization, phosphorylation of STING increases with increasing DNA length (described in the text on page 6, line 100 - 103).

3.) Can the authors speculate on the mechanism of saturation for the IFN response seen at higher DNA concentrations, which is proposed to occur downstream of TBK1 - i.e. have the authors looked at IRF3 phosphorylation or nuclear translocation?

As reviewer #2 suggests, we would hypothesize the saturation of the IFN pathway to take place at the level of IRF3 activity or at the level of IFN-receptor feedback signaling. These speculations have been added to the manuscript on page 7, line 112. However, we perceive further investigations of the saturation of the IFN induction pathway to be beyond the scope of this study.

4.) In Fig EV 2A, the blot for IFI16 ko cells shows lower cGAS levels, possibly explained by the lower levels of the loading control vinculin. This may be misleading, as it can hint towards an indirect role of IFI16 in DNA sensing via cGAS expression (which is unlikely to be true). An improved blot of wt and IFI16 ko cells with even loading control (and even cGAS levels, as shown previously in these cells) is recommended.

The blots for IFI16-deficient cells and control cells have been replaced with more equally loaded blots, now showing more equal cGAS levels (Fig EV3A).

Referee #3:

The authors have answered my concerns: the additional data with digestion products from two different regions of a plasmid (including a set based on a human gene), clearly establishes that the effect is not limited to that of PCR products, and is not limited to the bacterial sequences in the vector. The demonstration that all the transfection reagents testes also led to the same observation reinforces the concept that the contribution of this factor is likely negligible to the phenotype described - also seen in vitro with recombinant cGAS. The clarification that the length dependent effect is also visible at high dose, now included was also important. Altogether, the claims are now strongly supported by the data.

3rd Editorial Decision 10 July 2017

Thank you for the submission of your revised manuscript to our editorial offices. Before we can proceed with formal acceptance, these further editorial requests need to be addressed:

Regarding data quantification and statistics, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and indicate the test used to

calculate p-values in the respective figure legends. Please also add a short paragraph about the statistical testing to the methods section. Please provide statistical testing for all figure panels applicable (n>=3).

Please upload the abstract written in present tense.

Please remove the green highlights in the manuscript text, and replace "results" by "results and discussion" (or "results and conclusion").

I look forward to seeing a final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

3rd Revision –author's response 14 July 2017

We are pleased to learn that our manuscript is close to formal acceptance. Here, we submit the final version of our manuscript, in which we have complied with the editorial requests, including statistical analysis.

4rd Editorial Decision - acceptance 18 July 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Soren Paludan	
Journal Submitted to: EMBO Reports	
Manuscript Number: EMBOR-2017-44017	

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	As is common in cell-culture based experiments, three biological replicates from one experiment are shown in the graphs, representative of three (in rare cases 2) independent experiments. Power calculations were not performed. The number of biological replicates and independent experiments performed are mentioned in every figure legend.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For the experiments shown here, no data points were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization was performed.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was performed.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	In every figure legend, the statistical tests performed are described.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	When performing ANOVA, the Brown-Forsythe test was used to exclude significant differences in standard deviations.
Is there an estimate of variation within each group of data?	The error bars in the graphs show the standard deviation. This information is included in every figure legend.
Is the variance similar between the groups that are being statistically compared?	Yes, as tested with the Brown-Forsythe test.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Commercial antibodies were used as follows: p-TBK1 (CST D52C2), TBK1 (CST D1B4), STING (CST D2P2F; RnD AF6516), vinculin (Sigma V9131), cGAS (CST D1D3G), IFI16 (sc-8023), TREX1 (sc-271870), so mentioned in the manuscript on page 14.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The human monocyte-like THP-1 cells were obtained from ATCC (TIB-202). THP-1 cGAS-/-, STING-/-, B2M-/-, and IFI16-/- cells were previously generated using CRISPR-Cas9 technology (Holm et al., 2016; Jonsson et al., 2017). THP-1 Dual KO-TREX1 cells and THP-1 Dual cells were obtained from Invivogen. PBMCs were isolated from healthy blood donors (Blood Bank, Aarhus University Hospital). Human foreskin fibroblasts were obtained from ATCC (SCRC-1041). Cell origin is described on page 11 in the manuscript. In our lab, cell lines are routinely tested for presence of mycoplasma. No STR profiling was done.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Not applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No large data sets appropriate for deposition were generated in this study.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	No large data sets appropriate for deposition were generated in this study.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
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G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	To the best of our knowledge, no dual use is conceivable for our study.